Remarks/Arguments:

The specification is amended by inserting sequence identifiers for the amino acid sequences depicted as formulas (I), (II), and (III), as required in the instant Office Action.

The specification is also amended, hereby, to correct an obvious error. At the end of the paragraph bridging pages 31 and 32 the incorrect sequence "C-YTLRAPRSPKMVQGS-NH_2" appears for the sequence identifier "SEQ ID No. 16." Accordingly, the incorrect sequence is replaced by "C-YTLRAPRSPKMVQGSG-NH_2," i.e., the sequence that does correspond to "SEQ ID No. 16."

Claims 1-12, 14-31, and 33-35 are pending.

Claims 13 and 32 are cancelled without prejudice or disclaimer.

Present claims 5, 6, 15, 16, 17, 19, 21, 23, 24, 26, and 35 are currently amended by inserting appropriate sequence identifiers immediately following recited sequences. Claim 12 is amended by incorporating subject matter of claim 13 (dependent on claim 12 and, now, cancelled). Claim 31 is presently amended by incorporating subject matter of claim 32 (dependent on claim 31 and, now, cancelled).

Claims 11, 12, 30 and 31 are currently amended by inserting, at the end of each claim, subject matter supported in the specification (paragraph bridging pages 6 and 7, paragraph bridging pages 60 and 61, and figure 12). Claims 11, 12, 30, and 31 are also currently amended to more clearly define the subject invention; specifically, by rewriting the preamble (in each claim) to recite

"a human subject" and by rewriting the body (of each claim) with respect to the "biological sample" to recite "a biological sample of the human subject."

The specification and claims were objected to for allegedly failing to comply with PTO Rules governing disclosure of biological sequences. According to the statement of objection, compliance required inserting appropriate sequence identifiers in the specification and claims following each occurrence of the amino acid sequences depicted as formulas (I), (II), and (III).

As required, appropriate sequence identifiers are inserted into the specification and claims, as explained above. Accordingly, the subject application fully complies with PTO Rules governing disclosure of biological sequences and, as a result, withdrawal of the objection to the specification and claims appears to be in order.

Note is taken that the PTO maintains that the amino acid sequences depicted as formulas (I), (II), and (III) allegedly "do not appear in the Sequencing Listing for this application" and, therefor, the PTO argues "Applicant is required to submit a new sequence listing." The PTO is mistaken.

As explained above, corresponding sequence identifiers (from the sequence listing) are inserted immediately following the amino acid sequences of formulas (I), (II), and (III) in the specification and claims. Accordingly, applicants need not submit a new sequence listing, allegations to the contrary by the PTO, notwithstanding.

Claims 11-13 and 30-32 were rejected under 36 U.S.C. 112, second paragraph, as allegedly being indefinite. Reconsideration is requested in view of the changes to the claims effected hereby, taken in conjunction with the following remarks.

According to the statement of rejection the rejected claims are allegedly indefinite as "incomplete for omitting essential steps . . . . Claims 11, 12, 30, and 31 lack a correlation step. Claims 13 and 32 recite [a] correlation [step] but do not describe how the results of the assay allow for the determination."

With respect to claims 13 and 32 the rejection is rendered moot by cancellation of claims 13 and 32, hereby. Moreover, it should be pointed out that the PTO erroneously finds claims 13 and 32 indefinite for not describing "how the results of the assay allow for the determination" (emphasis added). Explaining *how* the invention is to be practiced is the function of the specification, not the claims; the function of the claims is to define the legal limits of the invention. *In re Roberts*, 176 USPQ 313, 315 (CCPA 1973).

With respect to rejected claims 11, 12, 30 and 31, the allegedly omitted "correlation step" is inserted by the instant amendment into each of the rejected claims. Accordingly, pursuant to the statement of rejection, the rejection as applied to claims 11, 12, 30 and 31 is overcome.

For the foregoing reasons, withdrawal of the rejection of claims 11-13 and 30-32 under §112, ¶ 2, appears to be in order for withdrawal.

Claim 29 was rejected under 35 U.S.C. 112, first paragraph, as allegedly lacking enablement. Reconsideration is requested.

Hyperdoma 3D4 deposited with the Collection Nationale de Cultures de Microorganisnes (SEE NCM) under a session number CNCM I-3073 (as disclosed at page 52 of the subject application), was deposited under the Budapest Treaty. Submitted herewith is a copy of the official

certificate of deposit, accompanied by an English language translation, as supporting evidence. Applicants are obtaining a declaration stating that the specific biological materials, deposited under Budapest Treaty, will be irrevocably and without restriction or condition released to the public upon the issuance of a patent. Accordingly, compliance with the deposit criteria set forth in the PTO Rules is met.

For the foregoing reasons, withdrawal of the rejection of claim 29 of the §112, ¶ 1, for allegedly lacking enablement is overcome. Withdrawal of the rejection appears to be in order.

Note is taken that the rejection of claim 29 mistakenly alleges that the instant specification does not "contain the accession number for the deposit, the date of the deposit, the name and address of the depository, and a description of the deposited materials sufficient to specifically identify it and to permit examination. The allegation is a mistake because the allegedly missing information is, in fact, disclosed in the instant specification (page 52, second paragraph).

Claims 11-13 and 30-33 were rejected under 35 U.S.C. 112, first paragraph, for allegedly lacking enablement. Reconsideration is requested.

According to the statement of rejection,

> the specification, while being enabling for [a] method of in vitro diagnosis of heart failure in a human, comprising bringing a blood sample into contact with an anti-proBNP (1-108) antibody as defined in claim 1, does not reasonably provide enablement for methods comprising all biological samples.

With all due respect, applicants cannot agree.

Satisfaction of §112, ¶ enablement does not require that applicants restrict the definition of "biological samples" in the rejected claims. The likelihood of wide distribution of proBNP (1-108)

would be inferred, but the skilled artisan, from the distribution of its metabolites BNP and NT-proBNP. That is, these metabolites have been found in urine (see the abstract and the introduction of Cortés *et al.*, *Eur J Heart Fail, 8*, 621-627, 2006, attached), cerebrospiral fluid (see the abstracts in both of Kaneko *et al.*, *Brain Research, 612*, 104-109, 1993, and Kirchhoff *et al.*, *J Neurotrauma, 23*, 943-949, 2006, attached), and brain tissues (see the first paragraph of the Blood-Brain Barrier section of the Discussion of Kirchhoff *et al.*, page 948).

Note is taken that the statement of rejection alleges that the rejected claims do not require that the biological sample "be obtained from the individual being tested." As explained, above, the rejected claims are rewritten to recite "a human subject" and "a biological sample of the human subject." This amendment to the claims renders this aspect of the rejection moot.

For the foregoing reasons, the rejection of claims 11-13 and 30-33 under §112, ¶ 1, for allegedly lacking enablement is overcome. Withdrawal of the rejection appears to be in order.

Request for Examiner's Initialed Form PTO 1449

According to the Office Action Summary page (of the instant Office Action) the Form PTO 1449 provided with applicants information disclosure statement filed September 14, 2005 (presumably marked to show whether the Examiner considered the references cited thereon) is an attachment to the Office Action. However, the Form PTO 1449 was not attached to the Office Action as received by applicant's undersigned attorneys of record.

Accordingly, the Examiner is requested to attach a copy of the missing, initialed Form PTO 1449 to the next Office communication.

Favorable action is requested.

Respectfully submitted,

s/William Player
William E. Player
Reg. No. 31,409

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TRAITE DE BUDAPEST SUR LA RECONNAISSANCE
INTERNATIONALE DU DEPOT DES MICRO-ORGANISMES
AUX FINS DE LA PROCEDURE EN MATIERE DE BREVETS

FORMULE INTERNATIONALE

DESTINATAIRE :

BIO-RAD Pasteur
3 Boulevard Raymond Poincaré
92430 MARNES-LA-COQUETTE

RECEPISSE EN CAS DE DEPOT INITIAL,
délivré en vertu de la règle 7.1 par
l'AUTORITE DE DEPOT INTERNATIONALE
identifiée au bas de cette page

NOM ET ADRESSE
DU DEPOSANT

I. IDENTIFICATION DU MICRO-ORGANISME

Référence d'identification donnée par le DEPOSANT :	Numéro d'ordre attribué par l'AUTORITE DE DEPOT INTERNATIONALE :
3D4	CNCM I-3073

II. DESCRIPTION SCIENTIFIQUE ET/OU DESIGNATION TAXONOMIQUE PROPOSEE

Le micro-organisme identifié sous chiffre I était accompagné :

[X] d'une description scientifique

[X] d'une désignation taxonomique proposée

(Cocher ce qui convient)

III. RECEPTION ET ACCEPTATION

La présente autorité de dépôt internationale accepte le micro-organisme identifié sous chiffre I, qu'elle a reçu le 31 juillet 2003 (date du dépôt initial) [1]

IV. RECEPTION D'UNE REQUETE EN CONVERSION

La présente autorité de dépôt internationale a reçu le micro-organisme identifié sous chiffre I le (date du dépôt initial)
et a reçu une requête en conversion du dépôt initial en dépôt conforme au Traité de Budapest le (date de réception de la requête en conversion)

V. AUTORITE DE DEPOT INTERNATIONALE

Nom : COLLECTION NATIONALE DE CULTURES DE MICROORGANISMES (CNCM) Adresse : Institut Pasteur 25, rue du Docteur Roux F-75724 Paris Cedex 15 (France)	Signature(s) de la (des) personne(s) compétente(s) pour représenter l'autorité de dépôt internationale ou de l'(des) employé(s) autorisé(s) : Georges Wagener Date : Paris, le 15 septembre 2003

1 En cas d'application de la règle 6.4.d), cette date est la date à laquelle le statut d'autorité de dépôt internationale a été acquis.

Formule BP/4 (page unique)

BUDAPEST TREATY ON THE INTERNATIONAL
RECOGNITION OF THE DEPOSIT OF MICROORGANISMS
FOR THE PURPOSES OF PATENT PROCEDURE

INTERNATIONAL FORM

TO

BIO-RAD Pasteur
3 Boulevard Raymond Poincaré
92430 MARNES-LA COQUETTE

RECEIPT IN THE CASE OF AN ORIGINAL DEPOSIT
issued pursuant to Rule 7.1 by the
INTERNATIONAL DEPOSITARY AUTHORITY
identified at the bottom of this page

NAME AND ADDRESS
OF DEPOSITOR

I. IDENTIFICATION OF THE MICROORGANISM	
Identification reference given by the DEPOSITOR: **3D4**	Accession number given by the INTERNATIONAL DEPOSITARY AUTHORITY: **CNCM I-3073**

II. SCIENTIFIC DESCRIPTION AND/OR PROPOSED TAXONOMIC DESIGNATION

The microorganism identified under I above was accompanied by:

[X] a scientific description

[X] a proposed taxonomic designation

(Mark with a cross where applicable)

III. RECEIPT AND ACCEPTANCE

This International Depositary Authority accepts the microorganism identified under I above, which was received by it on **July 31 2003** (date of the original deposit). [1]

IV. RECEIPT OF REQUEST FOR CONVERSION

The microorganism identified under I above was received by this International Depositary Authority on (date of the original deposit) and a request to convert the original deposit to a deposit under the Budapest Treaty was received by it on (date of receipt of request for conversion).

V. INTERNATIONAL DEPOSITARY AUTHORITY

Name: **COLLECTION NATIONALE DE CULTURE DE MICROORGANISMES (CNCM)**	Signature(s) of person(s) having the power to represent the International Depositary Authority or of authorized official(s): Georges Wagener
Address: **Institut Pasteur** **25, rue du Docteur Roux** **F-75724 Paris Cedex 15 (France)**	Date: Paris, September 15 2003

[1] Where Rule 6.4(d) applies, such date is the date on which the status of international depositary authority was acquired.

Form BP/4 (sole page)

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Intrathecal and Systemic Concentration of NT-proBNP in Patients with Severe Traumatic Brain Injury

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ABSTRACT

Outcome of patients suffering from traumatic brain injury (TBI) depends on the development of secondary brain damage. In this context, recent studies underlined the role of the natriuretic peptides—atrial natriuretic peptide and brain natriuretic peptide (BNP)—in aneurysmatic subarachnoidal hemorrhage (SAH). Especially BNP correlates with intracranial pressure and clinical outcome after SAH. Since its role in TBI remains unclear, the intracranial and systemic concentrations of *N*-terminal (NT)–proBNP were analyzed in patients suffering from severe TBI. We measured NT-proBNP levels in cerebrospinal fluid (CSF) and serum of 14 patients suffering from severe TBI (GCS ≤8 points) and 10 healthy control patients, using proBNP® assay (Roche® Diagnostics). Samples were collected after placement of a ventricular catheter, and at 12, 24, 48, and 72 h after TBI. CSF/serum albumin ratio ($Q < a$) was daily calculated. At 90 days after TBI, outcome was evaluated using the Glasgow Outcome Scale (GOS). In patients exhibiting a mean ICP of >15 mm Hg ($n = 6$), the serum (800 ± 150 pg/mL) and CSF levels (55 ± 9 pg/mL) of NT-proBNP were significantly increased after 24 h, as compared to patients with ICP ≤15 mm of Hg ($n = 8$) as well as to control group. However, Q_a as well as GOS did not significantly differ among both groups. For the first time, we evaluated intrathecal and systemic NT-proBNP concentrations in patients suffering from severe TBI. Interestingly, NT-proBNP in CSF and serum was significantly elevated in patients exhibiting an ICP of >15 mm Hg. Further studies are currently performed to elucidate the physiologic role of NT-proBNP in TBI.

Key words: amino terminal proBNP; cerebrospinal fluid; computerized tomography; intracranial pressure; NT-proBNP; traumatic brain injury

INTRODUCTION

TRAUMATIC BRAIN INJURY (TBI) is one of the major reasons for morbidity and mortality, especially in young trauma patients (Farin et al., 2004). The severity of the pathophysiological consequences is substantially influenced by the extent of the primary impact, including the mechanical injury to brain tissue and the development of secondary brain damage. Although the pathophysiological mechanisms responsible for the secondary brain damage remain incompletely understood so far, strong evidence has been raised that brain edema and swelling are major components. In this context, several potential activators of secondary brain swelling were

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identified, such as inflammatory destabilization and dysregulation of the cerebral water-sodium homeostasis in terms of cerebral salt wasting (CSW) (Ma et al., 2005). Recent studies on rat cerebral contusion models demonstrated that the dysregulation of cerebral water homeostasis might be influenced by the secretion of natriuretic proteins into the systemic circulation (atrial natriuretic peptide [ANP]) and the cerebral spinal fluid (brain natriuretic peptide [BNP]) (Fukui et al., 2003). Moreover, a significant correlation of intracranial pressure (ICP) and serum BNP levels after non-traumatic subarachnoidal hemorrhage was found (Berendes et al., 1997).

Although these results suggest that knowledge about the role of BNP in the development of brain edema might allow a more detailed insight into potential pathophysiological mechanisms leading to posttraumatic brain edema, its secretion pattern after TBI remains uncharacterized so far. Therefore, the aim of this pilot study was to investigate the systemic and intrathecal concentrations of *N*-terminal (NT)–proBNP in patients suffering from severe TBI and to compare the obtained results to quantitative markers of the blood–brain barrier (BBB) function and to elevation of ICP.

METHODS

Study Design and Patient Collective

The study was conducted between November 2003 and March 2005 at our university Level I trauma center; the study protocol was approved by the University Hospital Medical Ethics Board (reference no. 330/03). For control, cerebrospinal fluid (CSF) and serum were obtained from healthy patients, who obtained spinal anesthesia for an elective orthopedic intervention of the lower extremity. Patients with isolated TBI, presenting an initial Glasgow Coma Scale (GCS) score of ≤8 points (Teasdale et al., 1974) and radiological signs of intracranial brain injury upon admission (within 90 min of TBI)—that is, epidural or subdural hematoma, intracerebral or subarachnoid hemorrhage—were enrolled. Written informed consent was obtained from each patient when the patient returned to consciousness or, in case of remaining unconscious, when a next of kin or a legal representative was asked. Demographic and clinical information (i.e., gender, age, GCS) and history of systemic diseases was recorded using standardized data collection forms. Outcome was assessed using the Glasgow Outcome Score (GOS) at 6–12 months after trauma (Jennett et al., 1975). Exclusion criteria for eliminating bias caused by pre-existent alterations of NT-proBNP were previous cardiac, renal, hepatic, or endocrine disease, or receiving angiotensin-converting enzyme inhibitor drugs or diuretics prior to admission. According to ICP, patients were divided in two sub-collectives: group I exhibited a maximum ICP of <15 mm Hg for the entire observation period, whereas in group II minimum ICP values were >15 mm Hg. ICP and CPP were monitored permanently and recorded at each sampling point (n = 5 recorded observation points) according to the study protocol.

Clinical and Surgical Procedures

After initial stabilization and computerized tomography (CT), intraventricular drainage catheters (TraumaCath®; Integra® Neurosciences, Plainsboro, NJ) were placed in all patients within 90 ± 45min after admission for continuous ICP monitoring as well as for drainage of CSF. Subsequently, intracranial hematomas were surgically removed if necessary. After surgery, all patients were transferred to the intensive care unit (ICU) and treated in accordance with the guidelines of the brain trauma foundation: CPP was maintained at ≥60 mm Hg, ICP at ≤20 mm Hg. ICP therapy was immediately initialized comprising mild hyperventilation ($PACO_2$ ≤ 35 mm Hg, starting at the earliest time point 24 h after TBI), the use of mannitol boluses (maximum of 6 times/day, 0.25 g/kg body weight) as well as deep anesthesia using pentobarbital (Brain Trauma Foundation, 2000). In our therapy regime, steroid medication was not used. Intraventricular catheters were removed after the ICP levels remained at <15 mm Hg for at least 72 h.

Sampling Procedures

According to a serial protocol, 3 mL of drained intraventricular CSF and paired 5 mL of peripheral blood were collected. The first sampling time point was immediately after placement of the intraventricular drainage catheter (within 90 ± 45 min after TBI). In a standardized manner, all other CSF and serum samples were taken at 12, 24, 48, and 72 h after TBI. For the drainage of CSF, a continuous drainage method was chosen and used.

Analysis of NT-proBNP in Human Cerebrospinal Fluid and Serum

Concentration of NT-proBNP in CSF and serum was determined using a commercially available electrochemiluminescence immunoassay (ECLIA; Elecsys proBNP® assay; Roche® Diagnostics, Mannheim, Germany), as described previously (Jernberg et al., 2003). Synthetic human NT-proBNP was used for standardization.

Assessment of Blood–Brain Barrier Function

In order to determine whether NT-proBNP was released intrathecally or whether a passive leakage across

a dysfunctional BBB might be responsible for altered concentrations, the ratio of CSF and serum albumin (Q_a) was calculated for each observation point. According to Reiber and Felgenhauer (1987), Q_a is considered a sensitive parameter for the BBB dysfunction. The disturbance of the BBB was assessed as follows: Q_a values of <0.007 were regarded as normal, values of 0.007–0.01 as mild dysfunction, values of 0.01–0.02 as moderate dysfunction, and levels of >0.02 as severe dysfunction. Albumin levels were measured using standardized turbidimetric assay (Cobas Integra® Albumin; Roche® Diagnostics).

Statistical Analysis

The Sigma Stat® 3.0 software package (SPSS® Inc., Chicago) was employed for all statistical analysis. Statistical significance between groups was determined by independent two-tailed *t*-test. The Mann-Whitney *U*-test was used to analyze ordinal variables. In order to analyze the influence of functional parameters of the BBB on the NT-proBNP levels in CSF, linear regression analysis of NT-proBNP in CSF and Q_a values of all patients was performed. A *p*-value of <0.05 was considered to be statistically significant. Data are given as mean ± SEM.

RESULTS

Demographic and Clinical Data

A total number of 18 patients fulfilled the enclosing criteria. Four patients died within 24 h after TBI and had to be excluded due to the study protocol. A remainder of 14 patients survived the observation period (10 males, four females; mean age, 42 ± 4 years). Ten patients made uneventful recoveries, two died on days 5 and 6, and another two died on day 8 after TBI due to untreatable elevated ICP.

The major reasons for TBI were traffic accidents or falling from a height. Table 1 summarizes epidemiological data as well as brain injury pattern, GOS, and the mean ranges of ICP and Q_a for each individual. There

TABLE 1. EPIDEMIOLOGICAL DATA OF THE ENROLLED 14 TRAUMATIC BRAIN INJURY PATIENTS, ACCORDING TO GROUPS I AND II

Patient no.	*Injury pattern*	*Age*	*Sex*	*Interventions*	*Maximum ICP (mm Hg)*	*GOS (days post-trauma)*
Group I (n = 8), ICP ≤15 mm Hg						
I	SAH, SDH	32	f	—	13 ± 1	1 (8)
II	ICB	50	m	—	10 ± 2	5
III	Impression fracture, ICB, SAH	50	f	Fragment release	9 ± 1	5
IV	EDH, SAH, ICB, basal skull fracture	65	m	Osteoclastic craniectomy	12 ± 2	5
V	ICB	20	m	—	14 ± 1	4
VI	EDH, SAH	43	m	Osteoclastic craniectomy	9 ± 2	5
VII	Cerebellar ICB, SHD	48	m	Osteoclastic craniectomy	9 ± 1	4
VIII	SAH, SDH	52	m	—	12 ± 1	4
Group II (n = 6), ICP >15 mm Hg						
IX	Bitemporal SAH, contusion bleeding, SDH	82	m	—	39 ± 12	1 (8)
X	SDH, SAH, impression fracture, contusion bleeding	27	f	Osteoclastic craniectomy	18 ± 2	4
XI	SAH, ICB	58	m	Osteoclastic craniectomy	18 ± 2	4
XII	SAH, ICB, intracranial foreign body	23	m	Osteoclastic craniectomy	24 ± 1	1 (6)
XIII	SAH, SDH, basal skull fracture	41	f	Osteoclastic craniectomy	23 ± 1	1 (5)
XIV	SAH, SDH	35	m	Osteoclastic craniectomy	44 ± 7	3

Data are given concerning age in years, sex (m, male; f, female), brain injury pattern, surgical interventions, mean ± SEM of intracranial pressure (ICP) and the Glasgow Outcome Scale (GOS). SAH, subarachnoidal hemorrhage; SDH, subdural hematoma; ICB, intracerebral bleeding; EDH, epidural hematoma.

was no evidence of complications, such as intracranial hemorrhage or infection, due to insertion of the intraventricular catheter. No patient had evidence of transmural cardiac infarction or clinical evidence of heart failure at any stage of the study.

Patients of group I, $n = 8$ (ICP < 15 mm Hg) revealed a significantly lower mean ICP of 11 ± 1 mm Hg, compared to patients of group II ($n = 6$), who had a mean ICP of 28 ± 9 mm Hg. With respect to conservative ICP therapy, no significant differences in catecholamine dosage was observed. The groups did not differ significantly with respect to prognostic factors on admission such as gender, age, or CT classification. No differences concerning demographic parameters (gender, age) were observed between any of these groups. However, although patients in group I had a tendency of higher GOS of 4 ± 0.3 versus 2 ± 0.5 points in group II, the difference was not statistically significant.

Additionally, CSF and serum were obtained from 10 control patients (five males, five females; mean age, 40 ± 11 years), who obtained spinal anesthesia for an elective orthopedic surgery of the lower extremity. None of these patients had a history of chronic or acute inflammatory, or previous cardiac, renal, hepatic, or endocrine disease.

NT-proBNP Secretion in Cerebrospinal Fluid and Serum

In the control collective, the NT-proBNP levels in CSF accounted for 27 ± 2 pg/mL ($n = 10$). The CSF NT-proBNP levels of group I were significantly elevated on admission (38 ± 1 pg/mL, $n = 8$) and remained significantly increased during the entire observation period except for 48 h (32 ± 3) after TBI. In contrast, CSF NT-proBNP levels of group II were already on admission significantly higher (78 ± 12 pg/mL, $n = 6$) as compared to the control group as well as group I and remained significantly elevated until the end of the observation period (72 h, 132 ± 18). The dynamics of NT-proBNP levels in CSF are depicted for both groups in Figure 1.

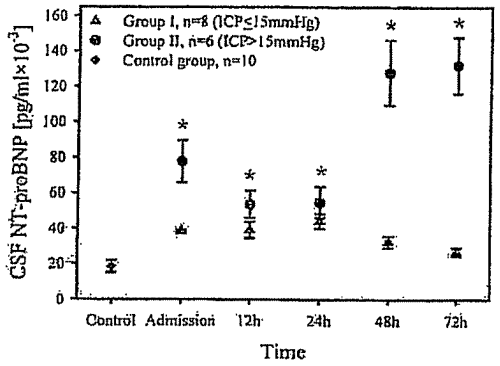

FIG. 1. Cerebrospinal fluid concentrations (CSF) of amino terminal pro-brain natriuretic peptide (NT-proBNP) in negative controls (squares, $n = 10$), followed by group I (triangle, $n = 8$) and group II (circle, $n = 6$) on admission, and at 12 h, 24 h, 48 h, and 72 h, given in pg/mL × 10^3. Data in mean ± SEM. *$p < 0.05$ versus group I, both patient groups are significantly elevated as compared to negative controls (except group I at 48 h). $p < 0.05$.

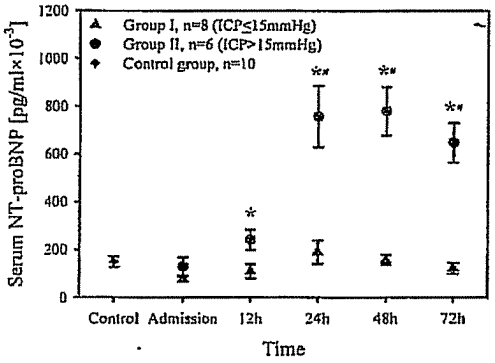

FIG. 2. Serum concentrations of amino terminal pro-brain natriuretic peptide (NT-proBNP) in negative controls (squares, $n = 10$), followed by group I (triangle, $n = 8$) and group II (circle, $n = 6$) on admission, and at 12 h, 24 h, 48 h, and 72 h, given in pg/mL × 10^3. Data in mean ± SEM. *$p < 0.05$ versus group I; #$p < 0.05$ versus negative controls.

In the serum of the control collective, the NT-proBNP concentration was 148 ± 21 pg/mL. At admission, serum levels in both groups were not different from control values (group I, 75 ± 10 pg/mL; group II, 127 ± 38 pg/mL). Starting from the 24-h observation point, serum levels were up to 10-fold higher as compared to CSF. Comparing both patient groups, NT-proBNP levels were significantly lower in group I as compared to group II, starting 24 h after TBI (group I, 188 ± 48 pg/mL; group II, 756 ± 128 pg/mL). Except a minimal transient elevation after 24 h, serum NT-proBNP levels of group I were always within normal ranges (Figure 2). In contrast, the dynamics of serum NT-proBNP in group II increased continuously up to 24 h and persisted significantly elevated up to the end of the study period (72 h, 647 ± 83 pg/mL). The difference concerning serum NT-proBNP between group I and II was significant after admission at all observation points.

Blood–Brain Barrier Function

The Q_a ratio was 0.010 ± 0.005 in group I and 0.021 ± 0.006 in group II on admission. During the observation

period, the Q_a ratio of group I remained mostly within ranges of a mild to moderate dysfunction (Fig. 3), whereas in group II an initially severe disturbed BBB improved continuously to a mild dysfunction 72 h after trauma (0.010 ± 0.005). No significant correlation between CSF NT-proBNP and Q_a values was detected using linear regression analysis.

DISCUSSION

We demonstrated a sequential analysis of NT-proBNP concentrations in CSF and systemic circulation of TBI patients. A significant increase of CSF and serum NT-proBNP concentration was found in patients exhibiting an increase of ICP, whereas Q_a as functional parameter of the BBB remained unaffected.

NT-proBNP

BNP was initially described as a biomarker for the identification of patients suffering from congestive heart failure (Maisel et al., 2002). Elevated serum levels were also found in patients with left ventricular dysfunction and ventricular pressure overload status such as pulmonary embolism, cor pulmonale and primary pulmonary hypertension (de Lemos et al., 2003). In the recent literature, there is also increasing evidence that BNP is significantly elevated in systemic inflammatory response syndrome (SIRS) (Charpentier et al., 2004). BNP was originally identified in extracts of porcine brain, as well as in the human hypothalamus and cardiac tissue (Saito et al., 1989; Takahashi et al., 1992). The protein is distributed as a proactive form of proBNP, comprising 108 amino acids, and is then cleaved into the biological active BNP (32 amino acids) and an inactive 76-residue *N*-terminal fragment (NT-proBNP). Although only BNP occurs to be biologically active in renal target cells, the cleaved NT-proBNP can be measured with higher sensitivity and accuracy due to the longer amino acid sequence (Mair et al., 2001). Since the NT-proBNP and BNP levels directly correspond to each other, the NT-proBNP was analyzed in this study (Lainchbury et al., 2003).

NT-proBNP in Traumatic Brain Injury Patients and Cerebrospinal Fluid

In our study, NT-proBNP in CSF was significantly elevated in TBI patients, exhibiting an ICP of >15 mm Hg, as compared to patients with an ICP of <15 mm Hg and to the control group. Our data are in contrast to a recent study, which investigated BNP secretion in CSF of patients suffering from SAH (Espiner et al., 2002). They found no significant differences as compared to negative control individuals. This discrepancy might be due to several factors, for example, the technique of measurement since BNP radioimmunoassay is known to be less sensitive in comparison to NT-proBNP analysis (Lainchbury

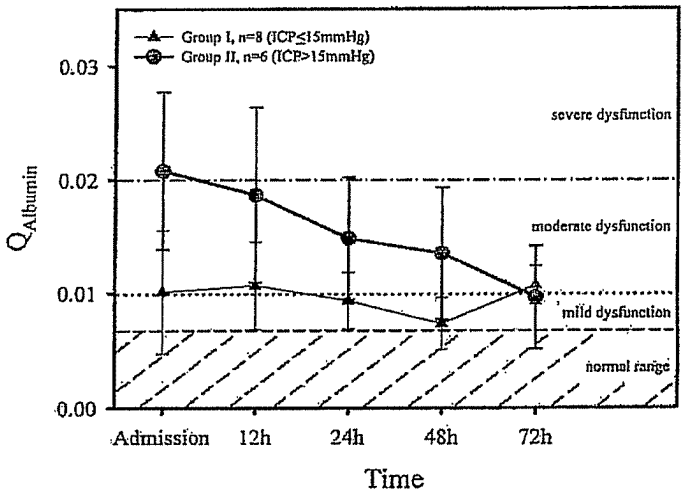

FIG. 3. $Q_{Albumin}$ (Q_a) in group I (triangle, $n = 8$), group II (circle, $n = 6$) on admission, at at 12 h, 24 h, 48 h, and 72 h. Q_a values of <0.007 (gray dashes) are regarded as normal, of 0.007–0.01 (gray dots) indicating a mild dysfunction, of 0.01–0.02 (gray dashes and dots) a moderate dysfunction, and of >0.02 a severe blood–brain barrier (BBB) dysfunction. No significant difference between group I and group II was present.

et al., 2003). Another factor might be the study design, including only a single time point of non-standardized measurement (with a median of 4 days of hemorrhage), and the lacking measurement of ICP.

NT-proBNP in Traumatic Brain Injury Patients and Serum

We demonstrated a significant increase of NT-proBNP in serum in TBI patients, exhibiting an ICP >15 mm Hg. For excluding elevation of NT-proBNP levels due to cardiac reasons, we excluded patients with previous cardiac ischemia or congestive heart disease (Berendes et al., 2001). Moreover, in all patients, troponin T as well as electrocardiography (ECG) was assessed on a daily basis. However, a new congestive heart failure could have been excluded only by invasive circulatory monitoring using pulmonary catheters, which was not feasible due to ethical reasons in terms of the invasive intervention for TBI patients.

Our data agree with other authors reporting an increase of BNP in serum of patients suffering from aneurysmatic SAH and concomitant increase of intracranial pressure (Berendes et al., 1997). Moreover, Sviri et al. (2003) described that BNP plasma concentrations after admission were significantly higher in patients suffering from severe bleeding as compared to patients with mild bleeding in a group of SAH patients. Therefore, he suggested that BNP release is associated with the intensity of brain tissue ischemia, reflecting increased biosynthesis and secretion from the ischemic brain tissue, especially from the hypothalamus in which BNP is widely distributed and synthesized. Other authors proposed that BNP increase might be caused by an iatrogenic fluid overload and pharmacologic therapy, inducing an increased cardiac load in terms of a general stress response to trauma or intensive care (Berendes et al., 1997). Nevertheless, all patients in our study received a standard treatment, including administration of fluid to keep a euvolemic or mildly hypervolemic status. Moreover, there were no significant differences concerning catecholamine dosage between both groups. Hence, the observed differences between both patient groups are more likely explained by differences in ICP than by treatment.

Blood–Brain Barrier

To further illuminate the source of NT-proBNP in CSF, we calculated the Reiber quotient (Q_a) as a functional parameter for the BBB, as it has been described previously by others (Morganti-Kossmann et al., 2001; Reiber et al., 1987). Thereby, we found no significant alteration between both groups and no correlation between a BBB dysfunction and increased CSF NT-proBNP levels. On the contrary, in group II, an improved function of the BBB after 72 h came along with the highest CSF NT-proBNP levels during the entire study period. Thus, the hypothesis that NT-proBNP is augmented from brain tissue is clearly supported. In the current literature, several studies described that severe TBI results in vascular damage and secondary release of vaso-active factors (Armstead, 1996; Morganti-Kossmann et al., 2001). In this respect, initial vascular damage might be responsible for increased BNP secretion by hypothalamic neurons. Moreover, Espiner et al. (2002) proposed a direct damage to the hypothalamus by the bleeding as the pathophysiological relevant mechanism for BNP release into serum. In particular, BNP is produced in the hypothalamus, and its production is mediated and induced by catecholamine triggers (Levin et al., 1998).

Taken together, it is suggested that NT-proBNP release is associated with the intensity of brain tissue ischemia, reflecting increased biosynthesis and secretion from the ischemic brain tissue, especially from the hypothalamus where BNP and ANP are widely distributed and synthesized.

CONCLUSION

A significant elevation of NT-proBNP was observed in the CSF and serum of patients suffering from severe TBI and pathologically elevated intracranial pressure. Due to no substantial signs of BBB damage measured by Q_a, it appears that serum NT-proBNP does not penetrate the CNS though leakage of BBB. Hence, cerebral synthesis might be a potential source of NT-proBNP and further studies are currently initiated to elucidate this phenomenon.

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Diagnostic and prognostic value of urine NT-proBNP levels in heart failure patients

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Abstract

Background: Plasma NT-proBNP levels are sensitive markers of ventricular dysfunction. However, studies of natriuretic peptides in urine are limited.

Aims: To compare urine and plasma NT-proBNP levels and to investigate the diagnostic and prognostic value of urine levels in heart failure (HF).

Methods: Urinary and plasma NT-proBNP levels were measured in 96 HF patients and 20 control subjects. The patients were functionally classified according to the NYHA criteria.

Results: Urine NT-proBNP was higher in HF patients than in control subjects (94±31 pg/ml vs. 67±6 pg/ml, $p<0.0001$), correlating with plasma NT-proBNP levels ($r=0.78$, $p<0.0001$). Urinary levels were elevated in the more severe functional classes and diminished in obese patients. Urine NT-proBNP was a good tool for diagnosis of HF, the area under the curve (AUC) being 0.96±0.02 ($p<0.0001$), and for predicting 12-month cardiac events ($p=0.011$). To determine the prognostic power of urinary NT-proBNP in detecting 12-month cardiac mortality, we obtained an AUC of 0.75±0.10 ($p=0.015$).

Conclusion: Urinary NT-proBNP, a relatively simple non-invasive test, is a new candidate marker for the diagnosis and evaluation of prognosis in HF and for the characterization of functional status in these patients.

Keywords: Natriuretic peptides; Urine; Heart failure

1. Introduction

Heart failure (HF) is a disease that is characterized by poor prognosis and quality of life. It is the most costly cardiovascular disorder in western countries, therefore, early identification and therapy for patients at high risk of developing HF or left ventricular dysfunction is required. Although echocardiography is the gold standard for diagnosis, it is not always readily available, especially in primary care [1]. Biochemical markers, such as the natriuretic peptide family, have been shown to be useful in the diagnosis of this syndrome. Plasma levels of brain natriuretic peptide (BNP) and its N-terminal precursor, N-terminal pro-BNP (NT-proBNP), are highly sensitive markers of ventricular hypertrophy [2,3] and/or left ventricular dysfunction [4–6], including congestive HF [7] and

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acute myocardial infarction [8]. In established disease, these biomarkers also offer prognostic value and may be useful to guide therapy [9].

The majority of studies on the diagnostic and prognostic potential of natriuretic peptides in HF have been performed on plasma samples [10]. However, assessment of the concentration of natriuretic peptides in urine, a non-invasive and simple test, may be useful in certain circumstances. Previous studies on the presence of natriuretic peptides in urine and their clinical significance are limited. The presence of C-type natriuretic peptide (CNP) has been demonstrated in the kidney and urine of patients with congestive HF [11]. In addition, urinary excretion of N-terminal pro-atrial natriuretic peptide (N-ANP) has been investigated in patients with kidney disease [12], BNP was described and its molecular form characterized in urine specimens of normal humans [13] and urine NT-proBNP has been determined in normal volunteers subjected to tilting and volume loading [14]. Furthermore, several studies have recently been published using urinary NT-proBNP levels as a diagnostic aid for left ventricular systolic dysfunction, using a non-competitive immunoluminometric assay [15,16], which is different from the assay employed in this study. These studies highlight the potential of this relatively simple urine test for the diagnosis of HF. However, the prognostic value of urinary NT-proBNP levels has not been studied to date.

Therefore, the aims of this study were to analyze the presence of NT-proBNP in human urine, to compare the urine levels with those found in plasma, and then use these results to investigate the potential diagnostic and prognostic value of urinary NT-proBNP in HF.

2. Methods

2.1. Patients

A total of 116 subjects were studied, of these 96 were consecutive HF patients and 20 were age- and gender-matched controls. Heart failure was diagnosed according to the European Society of Cardiology (ESC) HF criteria: electrocardiogram, chest X-ray and echo-Doppler study [17]. Aetiology of HF was multifactorial, as follows: ischaemic cardiomyopathy (45%), dilated cardiomyopathy (39%), hypertensive cardiomyopathy (13%) and valvular disease (3%). All patients were functionally classified according to the New York Heart Association (NYHA) criteria and were receiving standard medical treatment following the guidelines of the ESC [17]. Subjects in atrial fibrillation, with acute coronary syndromes, acute and chronic liver, pulmonary and renal diseases were excluded. Twenty-seven patients were obese with a body mass index (BMI) ≥ 30 kg/m^2.

All of the control subjects presented a normal echo-Doppler study, electrocardiogram and haematological and biochemical analyses.

All subjects gave informed consent to participate in the study, which was approved by the appropriate institutional review boards or ethics review committees of each study centre. The study was conducted in accordance with the guidelines of the Declaration of Helsinki.

2.2. Urine and blood sampling

Venous blood was collected by venipuncture with the subject supine having rested quietly for at least 30 min. Subjects also provided a urine sample, the first urine of the day [18,19]. After centrifugation at 1300 rpm and 4 °C for 10 min, urine and plasma samples were separated and stored in cryotubes at −80 °C until assayed. Before the analysis, the urine samples were centrifuged twice at 13,200 rpm at 4 °C for 30 min to avoid possible NT-proBNP measurement interferences produced by the precipitation of salts in urine.

2.3. NT-proBNP determination

NT-proBNP levels in serum and urine were determined in duplicate using an electrochemiluminescence immunoassay (Elecsys 2010 from Roche Diagnostics, Germany) based on the sandwich principle [20]. Results are expressed in pg/ml for both urine and blood samples. The lower detection limit was 5 pg/ml and the coefficient of intra-assay variation was 2.6%.

2.4. Echo-Doppler study

The echo-Doppler study was performed using the standard echocardiographic procedures of the hospitals involved in the study, as applied in routine clinical practice, with 2.5 MHz transducers. Cardiologists assessing left ventricular function were blinded to the results of the NT-proBNP assay. Two-dimensional images, Doppler spectrum and colour Doppler were stored on videotape and analyzed off-line at a central laboratory, using a computerized system (Eco-dat; Software Medicina SA). For each patient in regular rhythm, four consecutive beats were measured and averaged for each Doppler variable.

To obtain ejection fraction (EF), the area-length method was used and calculated as 100*[(telediastolic volume−telesystolic volume)/telediastolic volume]. By pulsed Doppler, peak flow velocity in early diastole (E wave) and during atrial contraction (A wave) was measured at valve level, calculating E/A ratio.

2.5. Statistical analysis

Results are presented as mean±SD. Results for each variable were tested for normality using the Kolmogorov Smirnov method. Data showing no normal distribution were compared using the Mann–Whitney test and categorical clinical variables were compared with Fisher's exact test. Plasma NT-proBNP levels were correlated with those in

urine using Spearman's rank correlation coefficient. Natriuretic peptide data were log-transformed before stepwise linear regression analysis to determine the independent predictors of urinary NT-proBNP levels. The discrimination of the best model was based on the principle of the least mean square and higher R-square. Regression analysis included sex, age, plasma NT-proBNP, serum creatinine, obesity (BMI>30 kg/cm), EF and *E/A* ratio as independent variables and urine NT-proBNP levels as dependent variable.

The relativity sensitivity, specificity and predictive value of urinary and plasma NT-proBNP levels for the absence or presence of HF were assessed by construction of receiver operating characteristic (ROC) curves. Logistic regression was performed on HF patients to evaluate the power of urine NT-proBNP in combination with EF, NYHA classes, age and obesity for prediction of 12-month events (mortality+cardiac admissions). ROC curve was also calculated to predict 12-month cardiac mortality from urinary NT-proBNP levels. The HF patients were divided into two groups based on urine peptide levels above and below 92.61 pg/ml to study differences in outcome rates using Fisher's exact test. This cut-off value was chosen based on the statistical results of the ROC curve, calculated to analyze the predictive value of urinary peptide levels in detecting cardiac mortality. All statistical analyses were performed using the Statistical Package for Social Sciences (SPSS 10.1) software (SPSS Inc., Chicago, Illinois). A p value <0.05 was considered significant for all parameters.

Table 1
Characteristics of heart failure patients

Variables	Patients (n=96)
Male sex (%)	71
Age (years)	66±12
SBP (mm Hg)	129±22
Heart rate (beats/min)	74±12
Serum creatinine (mg/dl)	1.22±0.7
Total cholesterol (mg/dl)	187±31
Na (mEq/l)	140±4
Haematocrit (%)	41±6
NYHA class I/ II/ III: (%)	9/ 70/ 21
Treatment (%)	
Diuretics	81
Beta blockers	61
Calcium-channel blockers	14
ACE inhibitors	72
Aldosterone antagonists	51
Digoxin	24
ARA II	17
History of hypertension (%)	58
Diabetes mellitus (%)	42
Smoking (%)	37
BMI (kg/m^2)	28±5
EF (%)	36±11
E/A	1.1±0.8

Data are presented as the mean value±SD or percentage of subjects.
ARA, angiotensin receptor antagonist; ACE, angiotensin-converting enzyme; BMI, body mass index; EF, ejection fraction; *E/A*, flow velocity in early diastole and during atrial contraction ratio; Na, sodium; NYHA, New York Heart Association; NT-proBNP, N-terminal pro-brain natriuretic peptide; SBP, systolic blood pressure.

Table 2
Comparison of urine and plasma NT-proBNP levels between heart failure patients and control subjects

Variables	Patients (n=96)	Controls (n=20)	p value
NT-proBNP levels (pg/ml):			
Plasma levels	1406±1821	36±24	<0.0001
Urinary levels	94±31	67±6	<0.0001

Data are presented as the mean value±SD.
NT-proBNP, N-terminal pro-brain natriuretic peptide.

3. Results

Clinical characteristics of patients are summarized in Table 1. Differences in urinary and plasma NT-proBNP levels between controls and patients were highly significant ($p<0.0001$) (Table 2). Furthermore, in HF patients plasma NT-proBNP levels were higher than urinary levels, but in control subjects peptide levels were more elevated in urine (Table 2). Urinary NT-proBNP levels showed good correlation with plasma NT-proBNP levels ($r=0.78$, $p<0.0001$) in our study population. In HF patients the correlation was $r=0.66$, $p<0.0001$, but in control subjects urine and plasma NT-proBNP levels showed no significant relationship.

The ROC curve of urine NT-proBNP for detection of HF yielded an area under the curve (AUC) of 0.96±0.02, $p<0.0001$ compared with the diagonal (Fig. 1). When a ROC curve was plotted for plasma NT-proBNP, a slightly higher AUC (0.98±0.01) was found (Fig. 1). From the ROC for urinary NT-proBNP, the optimal cut-off value (74.23 pg/ml) had a sensitivity and specificity of 93% and 95% for detection of HF, with positive and negative predictive values of 90% and 94%, respectively.

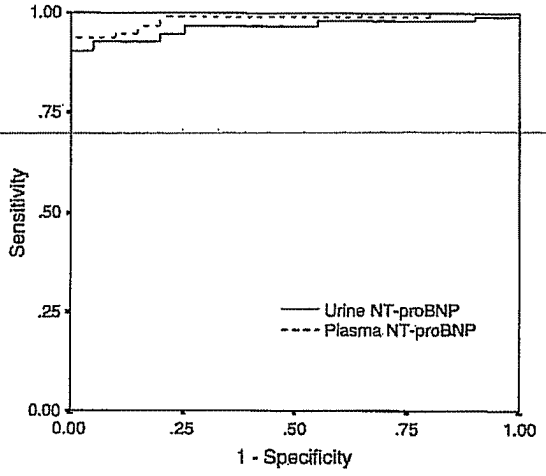

Fig. 1. Receiver operating characteristic curve of N-terminal pro-brain natriuretic peptide (NT-proBNP), urinary and plasma levels, for the detection of heart failure.

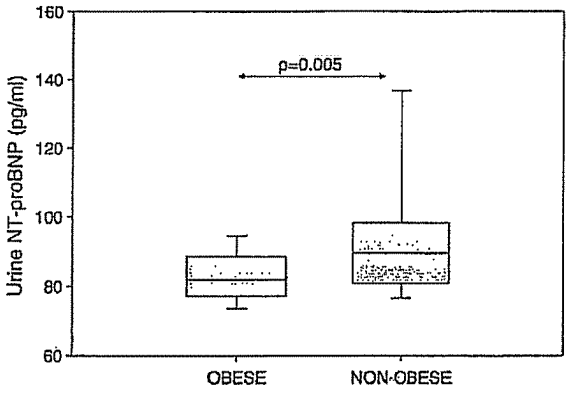

Fig. 2. Box plots showing urinary NT-proBNP (N-terminal pro-brain natriuretic peptide) levels in obese and non-obese heart failure patients. Boxes display median (horizontal bars), interquartile ranges (lower and upper limits of boxes) and 5th and 95th percentiles (error bars).

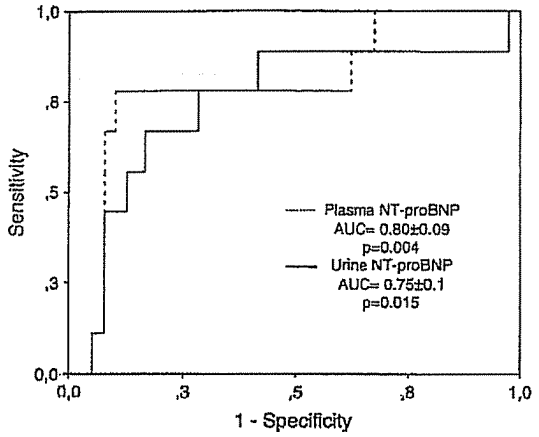

Fig. 4. Receiver operating characteristic curve of urinary and plasma N-terminal pro-brain natriuretic peptide (NT-proBNP) levels in predicting 12-month cardiac mortality in heart failure patients.

Recently, we have reported that obese subjects with heart failure have lower NT-proBNP plasma levels than non-obese HF patients [21]. In accordance with these findings, our current study found that obese patients had lower urine NT-proBNP levels than non-obese patients (84.7±9.8 vs 98.7±35.7 pg/ml, $p=0.005$) (Fig. 2). Furthermore, a multivariate linear regression analysis was used to test the independent predictive value of obesity on urine NT-proBNP levels in these patients. However, the best model associated with urine NT-proBNP levels did not include obesity as an independent predictor, but plasma NT-proBNP ($p<0.0001$) and plasma creatinine ($p=0.016$) accounted for an r^2 of 0.76.

Fig. 3 illustrates the distribution of NT-proBNP urine levels as a function of NYHA functional class (NYHA I: 77±10 pg/ml, NYHA II: 88±16 pg/ml, NYHA III: 123±51 pg/ml) ($p<0.0001$). The mean urinary level was significantly elevated in deteriorated NYHA classes and there were significant differences between NYHA I patients and control subjects (77±10 vs. 67±6, $p=0.025$). Results obtained in NT-proBNP plasma samples according to NYHA classes are similar to previous published studies (Fig. 3).

Furthermore, to investigate whether urinary NT-proBNP levels are independent predictors of 12-month events (mortality+cardiac admissions) a logistic regression was performed including EF, NYHA class, age and obesity, as well as urinary NT-proBNP levels. When the multivariate model was applied, urinary NT-proBNP level was a strong predictor of cardiac events ($p=0.011$), with an odds ratio

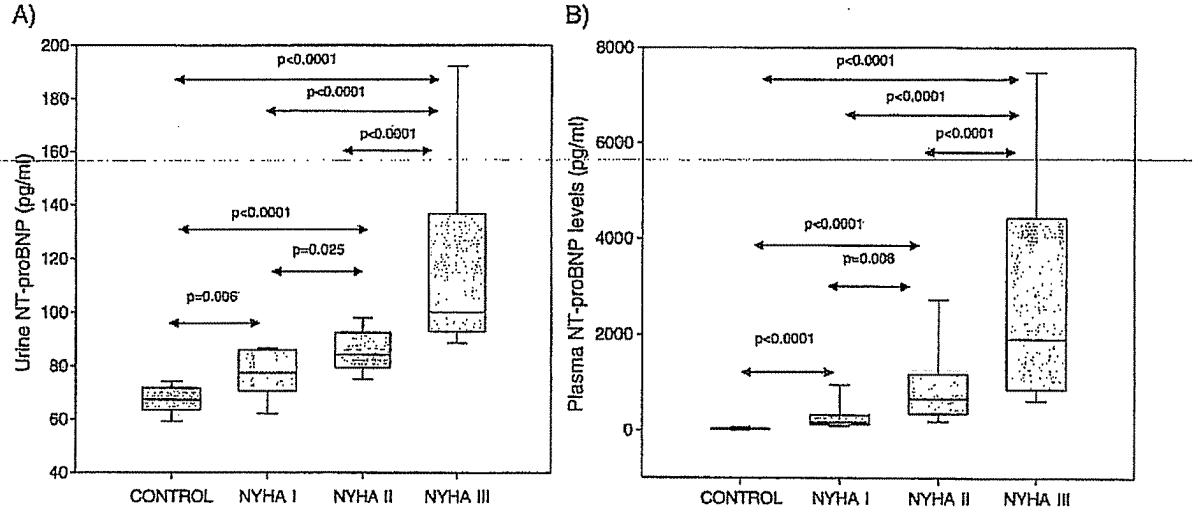

Fig. 3. Distribution of urinary (A) and plasma (B) NT-proBNP (N-terminal pro-brain natriuretic peptide) levels according to the degree of functional deterioration (New York Heart Association class). Boxes display median (horizontal bars), interquartile ranges (lower and upper limits of boxes) and 5th and 95th percentiles (error bars).

Table 3
The cardiac events for heart failure patients in based on a NT-proBNP cut-off value of 92.61 pg/ml

	NT-proBNP <92.61 pg/ml (n=65)	NT-proBNP >92.61 pg/ml (n=31)	p *value*
Combined cardiac events	14 (21%)	16 (52%)	0.006
Cardiac mortality	3 (4%)	7 (24%)	0.006

Data are expressed as absolute number of subjects. Combined cardiac events include (mortality+admissions). NT-proBNP, N-terminal pro-brain natriuretic peptide.

4.5 for the presence of cardiac event. Fig. 4 shows the ROC curve for urinary NT-proBNP to determine its prognostic power for detecting 12-month cardiac mortality, the AUC being 0.75±0.10 (p=0.015), plasma NT-proBNP levels showed a slightly higher AUC (0.80±0.09, p<0.015) for detecting mortality. Finally, to analyze HF patients according to urinary NT-proBNP levels, two groups were formed based on urinary NT-proBNP levels above and below 92.61 pg/ml. The 12-month-combined cardiac events (mortality+admissions) in the group of HF patients with NT-proBNP<92.61 pg/ml was 14 (21%) compared to that with NT-proBNP>92.61 pg/ml, which was 16 (52%) (p=0.006). When cardiac mortality was compared, significant differences were also obtained (p=0.006) (Table 3).

4. Discussion

Natriuretic peptides are strong diagnostic markers of ventricular hypertrophy [2,3] and/or left ventricular dysfunction [4–6]. However, previous studies on the presence of natriuretic peptides in urine and their clinical significance are limited [13–16]. The present study demonstrates that NT-proBNP levels are detectable in the urine of HF patients and control subjects, as reported in a previous study with healthy male volunteers [14]. Furthermore, higher NT-proBNP levels were found in plasma than in urine in 94% of HF patients, compared with only 10% of control subjects. These findings are contrary to previous reports [15,16]. These differences may be due to the fact that control subjects had a different peritubular renal circulation, resulting in little tubular reabsorption of this peptide into the bloodstream [22,23]. Moreover in the HF patients, urinary NT-proBNP levels showed good correlation with plasma levels, but the relationship did not improve when the urinary peptide levels were normalized according to the urinary creatinine levels [15,16], probably due to the different renal kinetics of these two molecules [24,25].

The influence of age, sex, systolic and diastolic ventricular function, serum creatinine and obesity on NT-proBNP plasma levels is well established [26–28]. To investigate how these variables affect urinary NT-proBNP levels, a stepwise linear regression was performed. NT-proBNP plasma levels and serum creatinine were independent predictors for urinary NT-proBNP levels in our HF patients. The dependence on plasma NT-proBNP and serum creatinine is not surprising, even at our creatinine values (1.21±0.7 mg/dl), because renal excretion is currently regarded as an important clearance mechanism [29]. Obesity was not an independent predictor of urinary NT-proBNP in this model, however, urinary NT-proBNP levels were diminished in obese patients (Fig. 2).

Previous studies have demonstrated the usefulness of plasma NT-proBNP levels in the diagnosis of HF [30,31] and left ventricular dysfunction [28,32–34]. Thus, it is not surprising that urinary NT-proBNP levels are also powerful predictors of HF.

Analysis of the area under the ROC curve of the current data suggests that under the design conditions of our study, a urinary NT-proBNP cut-off value of 74.23 pg/ml, discriminates HF patients from control subjects. Plasma NT-proBNP levels presented a slightly higher area under the ROC curve. Both testing methods showed similar sensitivities, specificities and negative predictive values and would be effective in the exclusion of a diagnosis of HF [34]. However, recently Ng et al. [16] showed low values of specificity and predictive positive value of plasma and urine NT-proBNP levels to diagnose left ventricular dysfunction in a community-screening study, using a non-competitive immunoluminometric assay to calculate the natriuretic peptide levels [15], which is different from the assay used in this study.

In the characterization of functional status in HF patients, mean urinary NT-proBNP levels were significantly elevated in the worse NYHA classes. Furthermore, differences were found between control subjects and patients in NYHA classes I, II and III.

An important issue in the determination of urine NT-proBNP is its prognostic value, and therefore, a logistic regression analysis was performed, including urinary NT-proBNP levels, EF, NYHA class, age and obesity, to predict 12-month outcome (mortality+cardiac admissions). Urinary NT-proBNP level was a strong predictor of cardiac events (p=0.011), with an odds ratio of 4.5 for the presence of a cardiac event. Furthermore, when a ROC curve for predicting 12-month cardiac mortality was constructed, urinary NT-proBNP levels showed an AUC of 0.75±0.10.

To analyze the cardiac events in HF patients using urinary NT-proBNP levels, two groups were formed according to the cut-off value (<92.61 pg/ml) obtained from the ROC curve used to predict cardiac mortality. The 12-month combined event rate was 21% in patients with low urinary NT-proBNP compared to 52% in those with NT-proBNP levels>92.61 pg/ml. The difference was 4% versus 24%, for mortality alone.

Further studies to evaluate the utility of urinary NT-proBNP levels in guiding treatment decisions in patients

with HF are now required. In particular, the utility of this non-invasive test must be proven in clinical practice (i.e., in a multicentre primary care study). This test may make natriuretic peptide levels more accessible for the general practitioner, with an additional potential benefit in terms of cost-effectiveness [35].

4.1. Limitations of the study

This study focuses on the analysis of NT-proBNP in the urine of HF patients, but the determination of the other natriuretic peptides such as BNP, and CNP, may also provide useful additional information. Another consideration is the fact that most of the patients in our study had moderate heart failure (NYHA II). A larger number of patients in worse functional classes may have given interesting additional results.

This study did not focus on a screening population; instead the test was applied to 96 patients with clearly confirmed HF and 20 controls, who were clearly free of HF. Thus, although the present data suggest the usefulness and applicability of this test, longer-term follow-up of a larger number of unselected patients is now required to confirm these findings.

In this study we used the first urine of the day for testing, as in previously reported studies [18,19]. It would have been interesting to confirm our findings with a full 24 h urine collection. However, due to the difficulty in collecting full 24 h urine in these ambulatory patients this was not done.

The Roche Elecsys 2010 analyzer is configured to test plasma NT-proBNP samples, and this could influence the urine NT-proBNP determinations. However, the good results obtained, when using the Roche Elecsys 2010 analyzer to calculate urine NT-proBNP for diagnostic and prognostic purposes, supports the utility of the measurements.

A common limitation in this kind of study is that the HF patients are receiving conventional therapy, and it is known that several drugs can reduce NT-proBNP levels. However, this study confirms that a high degree of neurohormonal activation persists in HF patients, even during standard therapy, and NT-proBNP values are useful for diagnostic and prognostic purposes.

4.2. Conclusions

This work shows that NT-proBNP can be determined in human urine and that urinary NT-proBNP is a new candidate marker for the diagnosis and prognosis of heart failure patients and for the characterization of functional status of these patients. This raises the possibility of using this relatively simple non-invasive test in primary care or in specific conditions where the collection of blood samples could be problematic. However, a large multicentre study to clarify the diagnostic power of urinary NT-proBNP levels in a general population is now required.

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C-type natriuretic peptide (CNP) is the major natriuretic peptide in human cerebrospinal fluid

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In order to investigate whether C-type natriuretic peptide (CNP) is present in human cerebrospinal fluid (CSF), we measured CNP-like immunoreactivity (-LI) in human CSF by specific radioimmunoassay (RIA) for CNP. We also measured atrial natriuretic peptide (ANP) and brain natriuretic peptide (BNP) concentrations in human CSF. ANP-LI, BNP-LI, and CNP-LI concentrations of CSF collected from fifteen patients without neurological disorders were 0.20 ± 0.13, 0.27 ± 0.10, and 2.13 ± 0.27 fmol/ml (mean ± S.D.), respectively. In fifteen patients with neurological disorders, ANP-LI, BNP-LI, and CNP-LI concentrations in CSF were 0.21 ± 0.18, 0.33 ± 0.19, and 2.09 ± 0.82 fmol/ml, respectively. Although ANP-LI and BNP-LI concentrations in plasma were much higher than those in CSF, CNP-LI was undetectable in plasma (less than 0.2 fmol/ml). These results demonstrate that three natriuretic peptides are present in CSF and that CNP is the major natriuretic peptide in human CSF. These results suggest that CNP in CSF is originated from and play important roles in the central nervous system.

INTRODUCTION

Atrial natriuretic peptide (ANP), isolated from cardiac atrium[5,28,29], is distributed not only in the heart but in the central nervous system (CNS)[6,14,16,21,22,30]. ANP is thought to play important roles in CNS as well as in the periphery to regulate blood pressure and body fluid homeostasis[16,30].

A second natriuretic peptide, brain natriuretic peptide (BNP), originally isolated from the porcine brain, has been demonstrated to exist mainly in the heart like ANP[1,2,4,15,20,24,25]. BNP has also central and peripheral actions similar to ANP[23].

C-type natriuretic peptide (CNP), also isolated from the porcine brain, is the third member of the natriuretic peptide family.[7,12,26] CNP, when injected intravenously into anesthetized rats, elicits natriuretic and hypotensive effects, but its potencies of these peripheral actions are weaker than ANP and BNP[26]. We and others have demonstrated that no significant amount of CNP mRNA and CNP are present in the heart, unlike ANP and BNP, and that considerable amounts of CNP are detectable throughout the brain. These findings indicate that CNP works mainly as a neuropeptide[9]. On the other hand, ANP and BNP function mainly as cardiac hormones[15,20,28,29].

Little is known about natriuretic peptides levels in human cerebrospinal fluid (CSF)[3,11,19,33]. In the present study, we have investigated concentrations of natriuretic peptide family in human CSF using specific radioimmunoassays (RIAs).

EXPERIMENTAL

Fifteen patients without neurological and cardiovascular disorders undergoing urological, gynecological or orthopaedic surgery by spinal anesthesia were studied (Table I). Six were men and nine were women with a

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TABLE I

Clinical profiles and natriuretic peptide levels in CSF and plasma of patients without neurological disorders

Case	Age / Sex	Op	CSF (fmol / ml)			plasma (fmol / ml)		
			ANP	BNP	CNP	ANP	BNP	CNP
1	28/F	CS	0.38	–	–	12.5	–	–
2	68/M	TUR	0.15	–	–	8.87	–	–
3	72/F	TUR	0.08	–	–	10.5	–	–
4	51/F	TKR	–	0.39	–	–	1.25	–
5	26/F	Biopsy	–	0.38	–	–	0.76	–
6	74/M	TUR	–	0.32	–	–	1.51	–
7	53/F	TAH	–	0.20	–	–	1.09	–
8	48/M	TUR	–	0.19	–	–	2.48	–
9	60/M	TKR	–	0.15	–	–	1.45	–
10	70/F	TKR	–	–	2.51	–	–	< 0.2
11	32/F	TAH	–	–	2.51	–	–	< 0.2
12	51/F	TKR	–	–	2.02	–	–	< 0.2
13	82/M	TUR	–	–	1.95	–	–	< 0.2
14	59/M	TUR	–	–	1.95	–	–	< 0.2
15	71/F	TUR	–	–	1.86	–	–	< 0.2
Total number			3	6	6	3	6	6
mean			0.20 +	0.27 #	2.13 *,**	10.6	1.42	< 0.2
±SD			±0.13	±0.10	±0.27	±1.51	±0.53	

Values are mean ± S.D. CS = ceaserian section; TUR = transurethral resection; TKR = total knee replacement; TAH = total abdominal hysterectomy.
+ $P < 0.01$ compared with ANP-LI level in plasma; # $P < 0.01$ compared with BNP-LI level in plasma; * $P < 0.01$ compared with ANP-LI level in CSF; ** $P < 0.01$ compared with BNP-LI level in CSF.

mean age of 56.3 years (26–82 years). CSF was obtained with a 23 gauge spinal needle at the induction of spinal anesthesia and blood sample was also obtained from peripheral vein at the same time.

Fifteen patients with various neurosurgical disorders, who had undergone spinal drainages for purposes of decompression or measurement of intracranial pressure, were also studied. Six were women and nine were men, with a mean age of 60 years (20–87 years). The underlying diseases were hypertensive intracerebral

TABLE II

Clinical profiles and natriuretic peptide levels in CSF and plasma of patients with neurological disorders

Case	Age / Sex	Disease	CSF (fmol / ml)			plasma (fmol / ml)		
			ANP	BNP	CNP	ANP	BNP	CNP
1	50/F	SAH	0.36	0.40	3.82	–	–	–
2	65/F	HIH	0.14	0.25	3.08	12.0	1.89	< 0.2
3	57/F	HIH	0.03	0.15	2.95	–	–	–
4	56/M	HIH	0.19	0.60	2.67	–	–	–
5	87/M	HIH	0.42	0.35	2.66	–	–	–
6	65/M	NPH	0.01	0.31	2.35	–	–	–
7	64/F	SAH	0.14	–	2.14	7.36	0.93	< 0.2
8	70/M	HI	0.22	–	1.98	–	–	–
9	68/F	HI	0.14	0.12	1.80	–	–	–
10	63/F	BT	0.30	–	1.78	12.1	0.97	< 0.2
11	62/M	I	0.28	–	1.64	–	–	–
12	63/M	HIH	0.12	0.31	1.36	6.91	1.07	< 0.2
13	66/M	HIH	0.02	0.70	1.33	11.2	1.66	< 0.2
14	20/M	BT	0.70	0.06	1.06	–	–	–
15	44/M	BT	0.03	0.42	0.67	3.02	1.51	< 0.2
Total number			15	11	15	6	6	6
Mean			0.21 +	0.33 #	2.09 *,**	8.77	1.34	< 0.2
±S.D.			±0.18	±0.19	±0.82	±3.33	±0.37	

Values are mean ± S.D. SAH = subarachnoid hemorrhage; HIH = hypertensive intracerebral hemorrhage; NPH = normal pressure hydrocephalus; HI = head injury; BT = brain tumor; I = infarction. + $P < 0.01$ compared with ANP-LI level in plasma; # $P < 0.01$ compared with BNP-LI level in plasma; * $P < 0.01$ compared with ANP-LI level in CSF; ** $P < 0.01$ compared with BNP-LI level in CSF.

hemorrhage in six, brain tumor in three, subarachnoid hemorrhage in two, head injury in two, cerebral infarction in one, and normal pressure hydrocephalus in one (Table II). CSF from the spinal drainage catheter was obtained. In 6 cases, CSF and blood sample from peripheral vein were obtained at the same time.

CSF and blood samples were transferred to ice-chilled tubes containing aprotinin (1,000 KIU/ml, Ohkura Pharmaceutical, Kyoto, Japan) and Na_2EDTA (1 mg/ml). Samples were centrifuged in 3,000 rpm at 4°C for 10 min, and supernatants of CSF and blood (plasma) were stored at −20 °C until extraction procedure.

The extraction of peptides was performed using Sep-Pak C_{18} cartridges (Waters Associates, Milford, MA, USA) as described previously[20,28,29]. 5 ml of CSF and 2–2.5 ml of plasma were applied to Sep-Pak C_{18} cartridges. The recovery rate of ANP, BNP and CNP added to CSF using Sep-Pak C_{18} cartridges was about 95%, 90% and 70%, respectively.

Measurement of CSF and plasma concentrations of ANP were performed using the RIA previously reported[17]. This RIA recognizes the C-terminal portion of α-human ANP (α-hANP). The minimal detectable quantity in the RIA is 0.3 fmol/tube. The cross-reactivities with human BNP (hBNP) and CNP were less than 0.01%[18].

Measurement of CSF and plasma concentrations of BNP were performed using the RIA previously described[15]. This RIA recognizes the ring structure of hBNP. The minimal detectable quantity of BNP in this RIA was 0.3 fmol/tube. The cross-reactivities with α-hANP and CNP were less than 0.005% and less than 0.01%, respectively[18].

Measurement of CSF and plasma concentrations of CNP were performed using the RIA previously described[9]. The minimal detectable quantity of CNP in this RIA was 2 fmol/tube. The cross-reactivities with α-hANP and hBNP were less than 0.2% and 0.01%, respectively[9].

High-performance gel permeation chromatography (HP-GPC) was performed on a TSK-GEL G2000 SW column (7.5 × 600 mm) (Toyo Soda, Tokyo, Japan) as already reported[14,17]. CSF obtained from patient of subarachnoid hemorrhage with spinal drainage was used for HP-GPC analysis. The flow rate was 0.3 ml/min and the fraction volume was 0.36 ml.

Data were expressed as mean ± S.D. Statistical analysis was performed using paired and unpaired Student's *t*-test and one-way analysis of variance when appropriate. Pearson's correlation was also used. *P* values of less than 5% were considered statistically significant.

RESULTS

Serial dilution of extracts of CSF gave curves parallel to the standard curves of RIA for ANP, BNP and CNP (Fig. 1). ANP, BNP and CNP were detected in human CSF.

ANP-like immunoreactivity (-LI), BNP-LI and CNP-LI concentrations in CSF of patients without neurological disorders were summarized in Table I. The CNP-LI concentration was the highest among three natriuretic peptides and approximately one order of magnitude higher than ANP-LI and BNP-LI concentrations ($P < 0.01$) (Fig. 2). There was no significant difference in the CSF concentration between ANP-LI and BNP-LI ($P > 0.1$).

ANP-LI, BNP-LI and CNP-LI concentrations in CSF of patients with neurological disorders were summarized in Table II. The CNP-LI concentration was also the highest among three natriuretic peptides ($P < 0.01$) (Fig. 2). There was no significant difference in the CSF

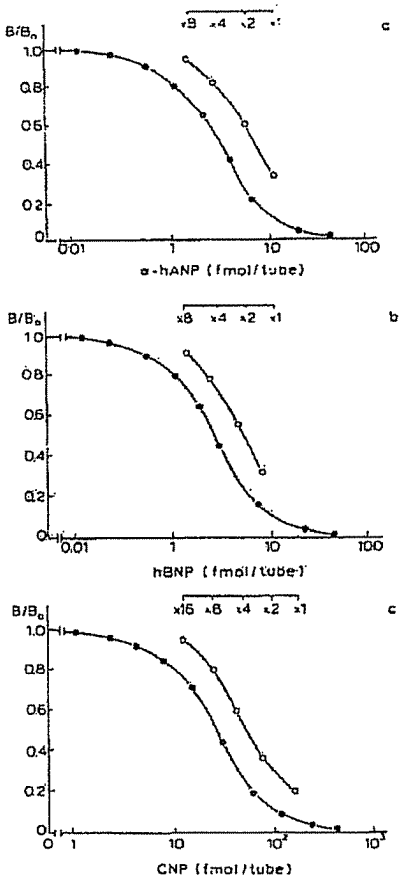

Fig. 1. a, b and c show typical standard curves of ANP, BNP and CNP(closed circles) and the dilution curve of the extracts from cerebrospinal fluid obtained from the patient who had undergone spinal drainage (open circles), respectively.

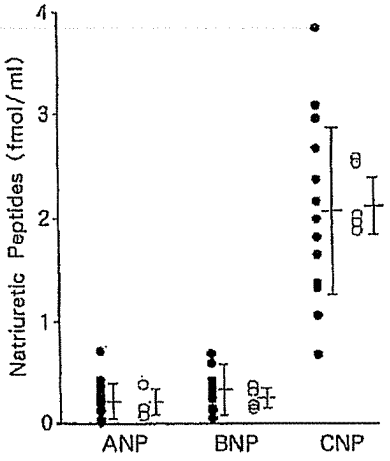

Fig. 2. Natriuretic peptides levels in CSF of patients with (Closed circles) and without (open circles) neurological disorders.

concentration between ANP-LI and BNP-LI ($P > 0.1$). No significant differences in values of natriuretic peptides concentrations in CSF were observed between patients with and without neurological disorders. Correlations in the CSF concentration between CNP-LI and ANP-LI ($r = 0.04$, $P > 0.1$), between CNP-LI and BNP-LI ($r = -0.04$, $P > 0.1$) or between ANP-LI and BNP-LI ($r = 0.54$, $P > 0.1$) were not significant.

CNP-LI concentrations in CSF of groups of patients with subarachnoid hemorrhage ($n = 2$), hypertensive intracerebral hemorrhage ($n = 6$), head injury ($n = 2$) and brain tumor ($n = 3$) were 2.98 ± 0.84, 2.34 ± 0.72, 1.89 ± 0.09 and 1.17 ± 0.46 fmol/ml, respectively. CNP-LI concentrations in CSF of groups of patients

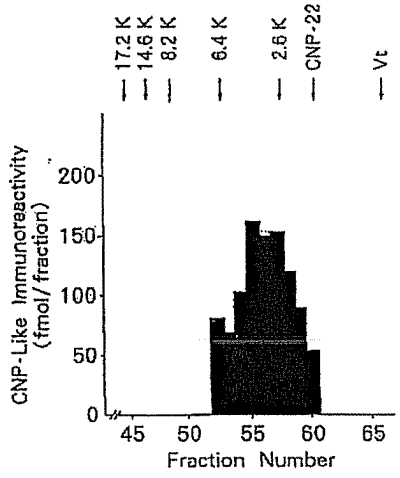

Fig. 3. HP-GPC profile of CNP-LI extracted from cerebrospinal fluid using a TSK-GEL G2000 SW column. Arrows indicate the elution position of synthetic CNP-22, a series of myoglobins of a polypeptide molecular kit (Pharmacia Fine Chemicals, Uppsala, Sweden), and total volume (V_t).

with subarachnoid hemorrhage and hypertensive intracerebral hemorrhage tended to be higher than that of group of patients with brain tumor, although this tendency was not observed in CSF concentrations of ANP-LI and BNP-LI.

ANP-LI and BNP-LI concentrations in plasma of both patients with and without neurological disorders were much higher than those in CSF ($P < 0.01$)(Tables I and II). However, CNP-LI concentrations in plasma were undetectable (< 0.2 fmol/ml). There were no significant correlations between the ANP-LI concentration in CSF and that in plasma ($r = 0.53$, $P > 0.1$) and between the BNP-LI concentration in CSF and that in plasma ($r = -0.13$, $P > 0.1$).

Fig. 3 shows a HP-GPC profile of the extract of CSF obtained from a patient with subarachnoid hemorrhage. CNP-LI was detected at elution positions of both CNP-22 and CNP-53, and the major peak was eluted at the position corresponding to the apparent molecular weight of 4–5 kDa, which was consistent with CNP-53.

DISCUSSION

The present study demonstrates that CNP is present in human CSF and that the concentration of CNP is the highest among natriuretic peptides in human CSF. The present finding is consistent with the recent observation that, in the human brain, the CNP concentration is one order of magnitude higher than ANP and BNP concentrations[13,18]. These results suggest that CNP plays important roles in the central nervous system.

The natriuretic peptide family possesses the conserved amino acid sequence, especially in the ring structure. Therefore, in order to measure exact concentrations of three natriuretic peptides, ANP, BNP and CNP, it is of importance to avoid cross-reactivities of each natriuretic peptide with the others in respective RIA. The cross-reactivities with ANP and BNP in the present RIA for CNP were less than 0.2% and 0.01%, respectively[9]. The cross-reactivities with BNP and CNP in RIA for ANP were also less than 0.01%[18]. And the cross-reactivities with ANP and CNP in RIA for BNP were less than 0.005% and 0.01%, respectively[18]. Thus, the RIAs for ANP, BNP and CNP used in the present study are specific for ANP, BNP and CNP, respectively.

In patients that CNP-LI concentrations both in CSF and in plasma were measured, the CNP-LI concentration in CSF was much higher than that in plasma, in which CNP was undetectable. CNP is known to be undetectable in rat plasma[9]. In addition, previous re-

ports demonstrated that ANP in CSF is originated from the central nervous system because systemically injected [^{125}I]α-ANP does not cross the blood–brain barrier[10]. It is unlikely, therefore, that BNP and CNP cross the blood–brain barrier. These results indicate that the source of CNP in CSF is not plasma but the central nervous system.

The HP-GPC profiles of CNP-LI in the extract of CSF revealed that both CNP-22 and CNP-53 exist in human CSF. Although the HP-GPC profile showed a relatively broad spectrum, the major peak is observed at the position corresponding to the apparent molecular weight of 4–5 kDa, corresponding to CNP-53. It is likely, therefore, that CNP-53 is the major molecular form of CNP in human CSF. This finding is compatible with the recent observations that CNP-53 is the major form of CNP-LI in the human brain[13,18].

CNP is not so potent as ANP and BNP in diuretic and hypotensive actions in the periphery[26]. However, since recent studies on ligand selectivity of natriuretic peptide receptors revealed that CNP is a selective ligand for ANP-B receptor[8,27], it is conceivable that CNP acts as a neuropeptide via the ANP-B receptor in the central nervous system.

In the present study, CNP-LI concentrations in CSF of patients with subarachnoid hemorrhage and hypertensive intracerebral hemorrhage tended to be higher than that of patients with brain tumor. However, since the numbers of patients in the present study were limited, further studies with large population are necessary to evaluate pathophysiological significance of CNP in these disorders.

Present results also demonstrate that the ANP-LI level in CSF is about 0.2 fmol/ml and much lower than that in plasma. There was no significant correlation in the ANP-LI concentration between CSF and plasma. In addition, there were no significant differences of ANP-LI concentrations in CSF among various neurological disorders. These results are compatible with those in previous reports[11,33]. But there are some reports showing that ANP concentrations in CSF were moderately elevated during the acute phase in patients with moderate to severe SAH[3,19]. The difference between the results may be explained by the difference in the sampling point of clinical course, because three cases with subarachnoid hemorrhage in this study were examined in chronic stage.

In the present study, the BNP-LI concentration was comparable to the ANP-LI concentration and much lower than that in plasma. Recently, Togashi et al. reported that the BNP concentration in human CSF collected by lumbar puncture is about 4 fmol/ml and higher than that in human plasma[31]. Their value in CSF is more than 10 times higher than ours. The cause of the difference is not clear at present. However, since the cross-reactivity of CNP in their RIA for BNP is not examined in their report, the high level of BNP-LI in CSF might be explained by CNP cross-reacted in their RIA for BNP. In addition, recent studies indicate that, in the human central nervous system, the CNP concentration is approximately one order of magnitude higher than ANP and BNP concentrations and that the BNP concentration is the lowest among three natriuretic peptides in the human brain[13,18].

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